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Upregulation of CD4 Expression during MHC Class II-Specific Positive Selection Is Essential for Error-free Lineage Choice

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SUMMARY

The lineage fate of developing thymocytes is determined by the persistence or cessation of T cell receptor (TCR) signaling during positive selection, with persistent TCR signaling required for CD4 lineage choice. We show here that transcriptional upregulation of CD4 expression is essential for error-free lineage choice during major histocompatibility complex class II (MHC II)-specific positive selection and is critical for error-free lineage choice in TCR-transgenic mice whose thymocytes compete for the identical selecting ligand. CD4 upregulation occurred for endogenously encoded CD4 coreceptors, but CD4 transgenes were downregulated during positive selection, disrupting MHC II-specific TCR signaling and causing lineage errors regardless of the absolute number or signaling strength of transgenic CD4 proteins. Thus, the kinetics of CD4 coreceptor expression during MHC II-specific positive selection determines the integrity of CD4 lineage choice, revealing an elegant symmetry between coreceptor kinetics and lineage choice.

INTRODUCTION

The fate of T cells developing in the thymus is determined by the specificity of their T cell antigen receptor (TCR). Thymocytes at the $CD4^+CD8^+$ (double positive [DP]) stage of development are the first cells in the thymus to express fully assembled αβTCR complexes and undergo either positive selection or cell death (Starr et al., 2003). Positively selected DP thymocytes ultimately differentiate into either $CD4^+$ helper or $CD8^+$ cytolytic T cells, with thymocytes bearing TCR specific for major histocompatibility complex class II (MHC II) ligands differentiating into $CD4^+$ helper T cells and thymocytes bearing TCR specific for MHC I ligands differentiating into $CD8^+$ cytolytic T cells (Starr et al., 2003). The cellular and molecular mechanisms by which positively selected thymocytes determine the ligand specificity of their TCR and their appropriate lineage fate have been the subject of intense investigation for years but have recently been clarified (Hedrick, 2008; Singer et al., 2008).

It is now understood that TCR-mediated positive-selection signals induce $CD4^+CD8^+$ double positive (DP) thymocytes to downregulate CD8 coreceptor expression and to convert into $CD4^+CD8^{lo}$ intermediate thymocytes (Bosselut et al., 2003; Brugnera et al., 2000). Positively selected $CD4^+CD8^{lo}$ intermediate thymocytes then differentiate into either CD4 or CD8 lineage T cells on the basis of whether TCR signaling persists or ceases (Brugnera et al., 2000; Sarafova et al., 2005; Singer, 2002; Singer and Bosselut, 2004; Yasutomo et al., 2000). Persistent TCR signaling induces intermediate thymocytes to upregulate expression of ThPOK, the CD4-lineage-specifying transcription factor, and to differentiate into $CD4^+$ helper T cells (He et al., 2005; He et al., 2008; Sun et al., 2005), whereas cessation of TCR signaling results in expression of RUNX3, a CD8 lineage-specifying transcription factor, and differentiation of intermediate thymocytes into $CD8^+$ cytolytic T cells (Setoguchi et al., 2008; Taniuchi et al., 2004). Thus, CD4 lineage choice requires persistent TCR signaling, whereas disrupted TCR signaling results in CD8 lineage choice (Sarafova et al., 2005; Singer, 2002; Singer and Bosselut, 2004; Singer et al., 2008).

Because CD4 and CD8 coreceptors stabilize and enhance MHC-specific TCR signals, quantitative changes in surface coreceptor expression during positive selection may affect TCR signaling duration and, consequently, CD4-CD8 lineage choice. Indeed, surface CD8 coreceptor expression declines on positively selected thymocytes as a result of decreased CD8 gene transcription, disrupting MHC I-specific TCR signaling and promoting CD8 lineage choice (Bosselut et al., 2003; Cibotti et al., 2000). In contrast to the kinetics of CD8 coreceptor expression during positive selection, which is well understood, it is not known whether surface CD4 coreceptor expression changes during positive selection and whether such changes affect the duration of MHC II-specific TCR signaling to contribute to CD4 lineage choice. Curiously, past experiments with transgenic CD4 coreceptors revealed that persistent CD4 coreceptor expression had a paradoxical impact on lineage choice in that it resulted in the aberrant generation of MHC II-specific $CD8^+$ T cells (Davis et al., 1993). These studies were interpreted as supportive of the stochastic-selection model of CD4-CD8 lineage choice, but subsequent experimental data make the existence of a stochastic mechanism underlying CD4-CD8

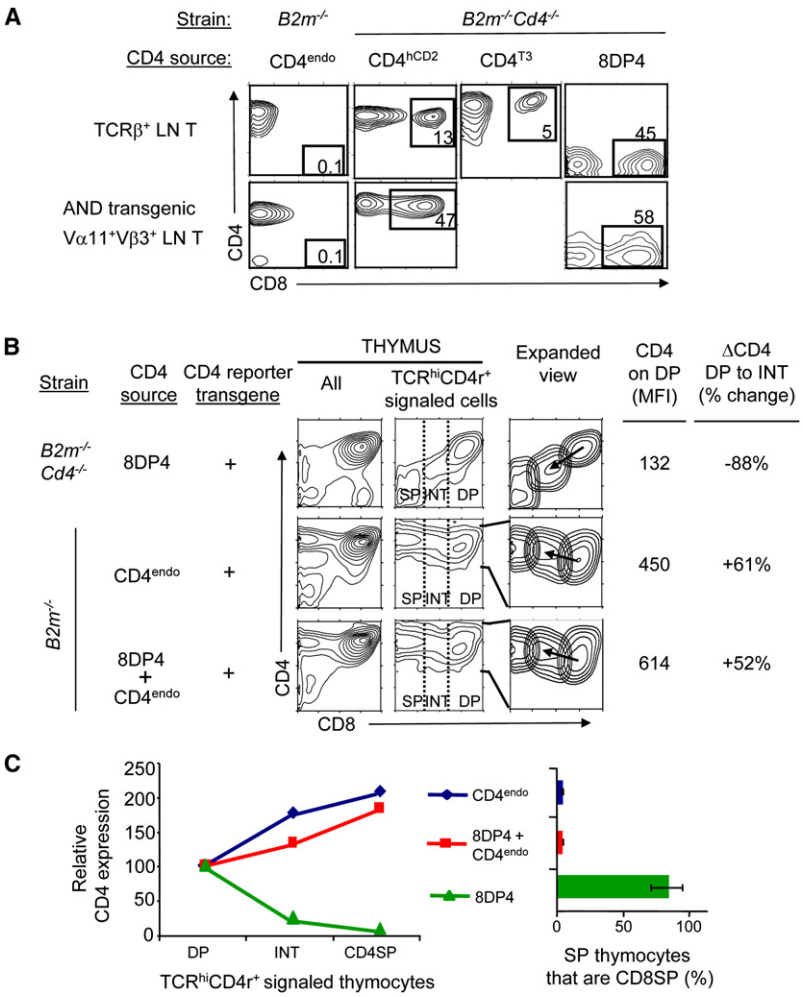

Figure 1. MHC II-Specific Lineage Choice Is Highly Error Prone in CD4 Transgenic Mice

(A) Phenotype of MHC II-selected LN T cells in mice expressing endogenous or transgenic CD4 coreceptor proteins. MHC II-selected lymph node (LN) T cells from three different strains of CD4 transgenic mice ($CD4^{hCD2}$, $CD4^{T3}$, and 8DP4) and from mice expressing endogenously encoded CD4 ($CD4^{endo}$) proteins were analyzed for CD4 and CD8 coreceptor expression (top row). Where indicated, mice also expressed the AND TCR transgene ($V\alpha11^+V\beta3^+$, bottom row). Boxes identify $CD8^+$ LN T cells and their relative frequencies. Data are representative of five independent experiments.

(B) Surface CD4 coreceptor expression changes during MHC II-specific positive selection in the thymus. Thymocytes from the indicated mice were analyzed for cell-surface expression of CD4, CD8α, TCRβ, and the CD4 reporter protein (CD4r) hCD2 (Figure S1). TCR-signaled thymocytes were defined as $TCR\beta^{hi}$ cells, with signaled DP thymocytes identified as $TCR^{hi}CD4r^+CD8^+$ cells, intermediate thymocytes (INT) identified as $TCR^{hi}CD4r^+CD8^{lo}$ cells, and CD4 single-positive (CD4SP) thymocytes identified as $TCR^{hi}CD4r^+CD8^-$ cells (middle column). Each signaled thymocyte population was then drawn individually, and histograms of all three thymocyte populations were overlaid on an expanded "y" scale, as indicated by the brackets (right column). CD4 surface expression on each thymocyte population was quantified as MFI with CD4 MFI on signaled DP thymocytes shown, and the change in CD4 surface expression (ΔCD4) between TCR-signaled DP and INT thymocytes was calculated and expressed as "% change" according to the following formula: % change = $100 \times (MFI^{INT} - MFI^{DP})/(MFI^{DP})$. Data are representative of four independent experiments.

(C) Kinetics of CD4 expression during MHC II-specific positive selection and its relationship to MHC II-specific lineage choice. CD4 expression on intermediate and CD4SP thymocytes was normalized to CD4 expression on signaled (i.e., $TCR\beta^{hi}$) DP thymocytes—which was set equal to 100%—from the same mouse (left panel). The frequency (± standard error [SE]) of SP thymocytes that were CD8SP in each mouse is displayed (right panel). The numbers of mice analyzed per strain were as follows: $CD4^{endo}$ = 10, 8DP4 = 10, and 8DP4+$CD4^{endo}$ = 2.

lineage choice no longer tenable (Adoro et al., 2008; Itano and Robey, 2000; Singer et al., 2008). Consequently, the paradoxical observation that expression of transgenic CD4 coreceptors induced a number of MHC II-specific thymocytes to differentiate into $CD8^+$ T cells remains unexplained.

The present study was undertaken to determine whether surface CD4 coreceptor expression undergoes changes during MHC II-specific positive selection and whether such changes affect lineage choice. We now report that endogenous CD4 coreceptor expression is in fact upregulated on MHC II-signaled thymocytes during positive selection and that such CD4 upregulation is essential for error-free lineage choice, regardless of the absolute number or signaling strength of the CD4 coreceptors. In the absence of CD4 upregulation, MHC II-specific lineage choice is highly error prone, a situation exacerbated in TCR transgenic mice whose thymocytes compete for a single selecting ligand. Thus, the kinetics of CD4 coreceptor expression during positive selection determines the integrity of CD4 lineage choice, complementing what is known about the kinetics of CD8 coreceptor expression to reveal an elegant symmetry between coreceptor kinetics and lineage choice.

RESULTS

To examine changes in CD4 coreceptor expression during MHC II-specific positive selection and their effect on MHC II-specific lineage choice, we compared MHC II-specific selection in mice that expressed CD4 coreceptor proteins under the control of either endogenous or transgenic transcriptional regulatory elements (Figure 1). To generate experimental mice for this study, we introduced transgenes encoding CD4 protein under the control of the human CD2 promoter ($CD4^{hCD2}$) (Van Laethem et al., 2007), the human CD3δ promoter ($CD4^{T3}$) (Davis et al., 1993; Lee et al., 1992), or the murine $E8_{III}$ enhancer plus CD8α promoter (8DP4) (Ellmeier et al., 1998; Sarafova et al., 2005) into $B2m^{-/-}Cd4^{-/-}$ so that only MHC II-selecting elements and only transgenic CD4 coreceptor proteins were expressed in the thymus (see Table S1 available online).

In nontransgenic $B2m^{-/-}$ mice expressing endogenously encoded CD4 proteins ($CD4^{endo}$), MHC II-specific selection resulted exclusively in $CD4^+$ T cells, indicating that MHC II-specific lineage choice was error free (Figure 1A, upper-left panel). In contrast, MHC II-specific lineage choice in $CD4^{hCD2}$ and $CD4^{T3}$ transgenic mice was error prone given that they contained substantial frequencies (13% and 5%) of $CD8^+$ lymph node (LN) T cells (Figure 1A, upper-middle panels). $CD8^+$ LN T cells in $CD4^{hCD2}$ and $CD4^{T3}$ mice appeared as $CD4^+CD8^+$ because transgenic hCD2 and hCD3δ transcriptional control elements remained active in $CD8^+$ T cells (Figure 1A, upper-middle panels). Notably, lineage errors did not require persistent CD4 transgene expression because MHC II-specific selection was even more error prone in a transgenic mouse (8DP4) in which transgenic CD4 expression was terminated early during positive selection (Figure 1A, upper-right panel). Error-prone MHC II-specific lineage differentiation in 8DP4 mice resulted in mature T cells that were phenotypically $CD4^-$ and appeared as either $CD4^-CD8^-$ or $CD4^-CD8^+$ cells (Sarafova et al., 2005). Thus, MHC II-specific lineage choice was error free in nontransgenic mice but was error prone in all three of the CD4 transgenic mice examined despite marked differences in duration of CD4 transgene expression. Similarly, MHC II-specific lineage choice by monoclonal AND thymocytes (Kaye et al., 1989), which are $V\alpha11^+V\beta3^+$, resulted only in $CD4^+$ T cells when AND thymocytes expressed endogenously encoded CD4 coreceptor proteins but was highly error prone when AND thymocytes expressed transgene-encoded CD4 coreceptor proteins (Figure 1A, bottom panels).

To understand the basis for error-free versus error-prone CD4 lineage choice, we considered that CD4 lineage choice requires MHC II-specific TCR signaling to persist during differentiation of signaled DP thymocytes into $CD4^+CD8^{lo}$ intermediate thymocytes, whereas any disruption in TCR signaling during this developmental step results in CD8 lineage choice (Brugnera et al., 2000; Singer, 2002). Consequently, we quantified changes in surface CD4 expression during differentiation of signaled DP thymocytes into $CD4^+8^{lo}$ intermediate cells. We identified signaled DP thymocytes and intermediate cells without relying on surface CD4 coreceptor expression by introducing into all mice a CD4 reporter (CD4r) transgene that reports endogenous *Cd4* transcriptional activity by surface expression of hCD2 proteins (Figure S1) (Sawada et al., 1994). Specifically, MHC II-signaled DP thymocytes were identified as $TCR^{hi}CD4r^+CD8^+$ cells; intermediate thymocytes were identified as $TCR^{hi}CD4r^+CD8^{lo}$ cells; and CD4SP thymocytes were identified as $TCR^{hi}CD4r^+CD8^-$ cells.

We first examined thymocytes from 8DP4 mice because the 8DP4 transgene was transcriptionally regulated by $E8_{III}$-CD8α enhancer elements so that TCR signaling downregulated CD4 surface expression and disrupted MHC II signaling during differentiation of DP into intermediate thymocytes (Sarafova et al., 2005). As shown in a representative experiment, surface expression of 8DP4-encoded CD4 proteins was dramatically reduced (−88%) during differentiation of signaled DP into intermediate thymocytes as CD4 mean fluorescent intensity (MFI) declined from 132 to 16 (Figure 1B, upper panels), a change best appreciated in the expanded view that was generated by gating individually on each thymocyte subset and overlaying their profiles (Figure 1B, upper-right panel). In marked contrast to 8DP4-encoded CD4 proteins, surface expression of endogenously encoded CD4 proteins substantially increased (+61%) during differentiation of signaled DP into intermediate thymocytes as CD4 MFI increased from 450 to 725 (Figure 1B, middle panels).

To determine whether such changes in CD4 expression during MHC II-specific positive selection affected the integrity of CD4 lineage choice, we assessed the frequency of MHC II-selected $CD8^+$ single-positive (SP) thymocytes (Figure 1C, right). We found that $B2m^{-/-}$ mice expressing 8DP4-encoded CD4 proteins contained a high frequency of MHC II-selected CD8SP thymocytes, whereas $B2m^{-/-}$ mice expressing endogenously encoded CD4 proteins were essentially devoid of CD8SP thymocytes (Figure 1C, right). Thus, endogenous CD4 surface expression *increased* during differentiation of MHC II-signaled DP into intermediate thymocytes and was associated with error-free CD4 lineage choice, whereas 8DP4-encoded CD4 surface expression decreased on MHC II-signaled thymocytes and was associated with error-prone CD4 lineage choice (Figure 1C, left and right panels).

To determine whether CD4 lineage choice was error prone in 8DP4 mice because CD4 expression was downregulated on MHC II-signaled thymocytes and not because of 8DP4 transgene expression per se, we generated $B2m^{-/-}$ mice that expressed both the 8DP4 transgene and endogenously encoded CD4 proteins (referred to as $8DP4+CD4^{endo}$ mice). Examination of $8DP4+CD4^{endo}$ thymocytes mice revealed that surface CD4 expression increased (+52%) during differentiation of MHC II-signaled DP into intermediate thymocytes (Figure 1B, last row) because upregulation of endogenous CD4 proteins more than compensated for decreased expression of 8DP4-encoded CD4 proteins during MHC II-specific positive selection given that CD4 MFI increased from 614 to 933 (Figure 1B, last row). Importantly, we found that $8DP4+CD4^{endo}$ mice were essentially devoid of CD8SP thymocytes (Figure 1C), revealing that CD4 lineage choice in $8DP4+CD4^{endo}$ mice was error free despite expression of the 8DP4 transgene.

Basis for Error-Prone CD4 Lineage Choice in CD4 Transgenic Mice

On the basis of our findings in 8DP4 mice that the kinetics of CD4 expression during MHC II-specific positive selection affects CD4 lineage choice, we predicted that error-prone CD4 lineage choice in CD4 transgenic mice might be due to a failure of transgenic CD4 proteins to be upregulated during MHC II-specific positive selection. To assess this prediction, we quantified changes in surface CD4 expression on DP and intermediate thymocytes from $CD4^{hCD2}$ and $CD4^{T3}$ transgenic mice, including an independently derived subline of $CD4^{T3}$ ($CD4^{T3}L2$) with lower overall CD4 protein expression (Figure 2A, and Figure S2). Although surface expression of endogenously encoded CD4 proteins increased during differentiation of signaled DP into intermediate thymocytes, transgene-encoded CD4 expression decreased in $CD4^{hCD2}$, $CD4^{T3}$, and $CD4^{T3}L2$ mice (Figure 2A). A summary of changes in CD4 expression during differentiation of MHC II-signaled DP thymocytes from the various mouse strains is displayed (Figure 2B, upper-left panel), along with frequencies of αβ LN T cells that had adopted the incorrect CD8 lineage fate (Figure 2B, right panel). In fulfillment of our

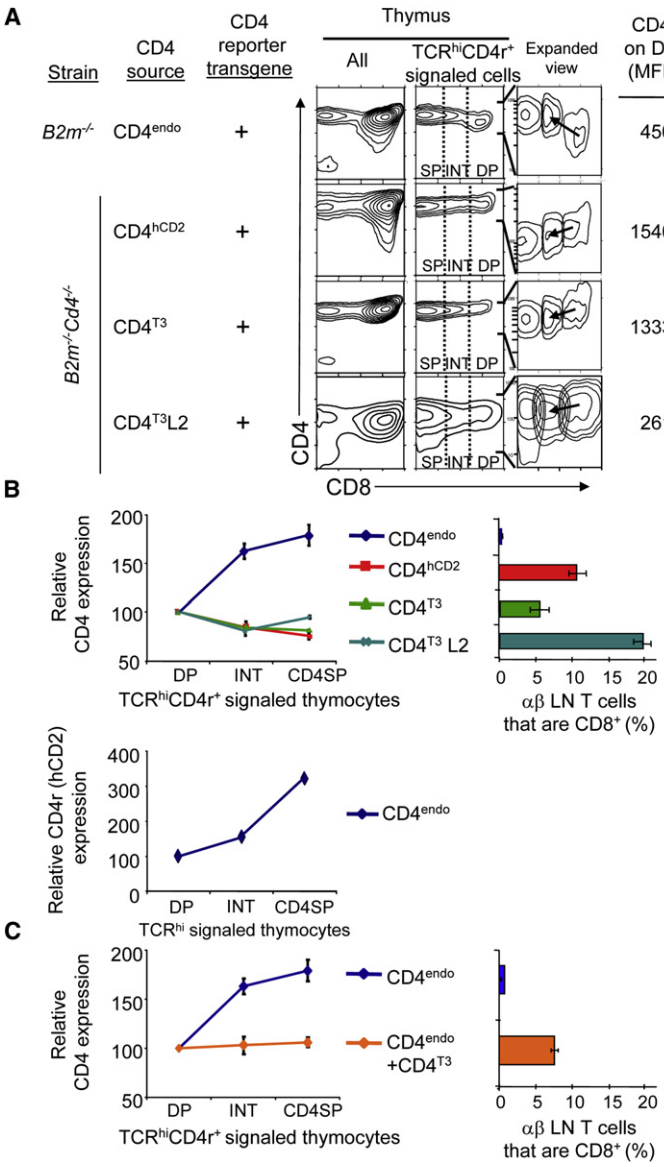

Figure 2. Coreceptor Kinetics and Lineage Choice in CD4 Transgenic Mice

(A) Quantitation of CD4 expression during MHC II-specific positive selection. Thymocytes were analyzed for cell-surface expression of CD4, CD8α, TCRβ, and the CD4 reporter protein (CD4r) (see Figures S2–S4). CD4 surface expression on signaled ($TCR\beta^{hi}$) DP, INT, and CD4SP thymocyte populations in each mouse were analyzed as in Figure 1B, with relative changes in CD4 surface expression between TCR-signaled DP and intermediate thymocytes quantitated as % change. Data are representative of four independent experiments.

(B) Coreceptor kinetics and its relationship to MHC II-specific lineage choice in CD4 transgenic mice. Surface expression of CD4 (± SE) (left upper panel) and hCD2 reporter protein (left lower panel) on intermediate and CD4SP thymocytes from individual mice were expressed relative to CD4 expression on signaled ($TCR\beta^{hi}$) DP thymocytes—which was set equal to 100% and so does not vary—from each individual mouse (left upper panel). Error-prone lineage choice is indicated by the frequency (±SE) of LN T cells that are $CD8^+$ for each strain (right panel). The numbers of mice analyzed per strain were as follows: $CD4^{endo}$ = 10, $CD4^{hCD2}$ = 7, $CD4^{T3}$L1 = 6, and $CD4^{T3}$L2 = 4.

(C) Coreceptor kinetics and its relationship to MHC II-specific lineage choice in $B2m^{-/-}$ mice expressing $CD4^{endo}$ and $CD4^{T3}$ proteins. Surface expression of CD4 (± SE) (left panel) on intermediate and CD4SP thymocytes from individual mice were expressed relative to CD4 expression on signaled ($TCR\beta^{hi}$) DP thymocytes—which was set equal to 100%—from each individual mouse (left panel) (see Figures S2–S4). Error-prone lineage choice is indicated by the frequency (± SE) of LN T cells that are $CD8^+$ for each strain (right panel). The numbers of mice analyzed per strain were as follows: $CD4^{endo}$ = 10 and $CD4^{endo}$+$CD4^{T3}$ = 4.

prediction, this analysis documented that, whereas surface expression of endogenously encoded CD4 proteins increased during differentiation of MHC II-signaled DP thymocytes into intermediate cells and resulted in error-free lineage choice, surface expression of transgene-encoded CD4 proteins decreased and resulted in error-prone MHC II-specific lineage choices (Figure 2B). Surprisingly, MHC II-specific lineage choice was error prone in all CD4 transgenic mice even though overall CD4 expression on transgenic thymocytes varied widely, ranging from relatively high CD4 expression ($CD4^{hCD2}$) to intermediate CD4 ($CD4^{T3}$) to low CD4 expression ($CD4^{T3}$L2) (Figure 2B, Figure S2B). Thus, regardless of the overall quantity of CD4 coreceptor expression on thymocytes, downregulation of surface CD4 expression during differentiation of signaled DP into intermediate thymocytes results in error-prone MHC II-specific lineage choice.

We also determined CD4 expression on signaled DP and intermediate thymocytes from $CD4^{T3}$ ($B2m^{-/-}$) mice expressing both endogenous and transgenic CD4 proteins, because we thought that transcriptional upregulation of endogenous CD4 proteins during MHC II-specific positive selection might be counterbalanced by downregulation of transgenic $CD4^{T3}$ proteins. In fact, in $CD4^{T3}$ ($B2m^{-/-}$) mice, overall CD4 surface expression remained essentially unchanged during differentiation of signaled DP into intermediate thymocytes, as well as during their subsequent differentiation into CD4SP cells (Figure 2C, left panel). MHC II-specific lineage choice in these mice was also error prone as revealed by both the frequency of αβ LN T cells that were $CD8^+$ (Figure 2C, right panel) and the presence in the thymus of $HSA^-TCR\beta^{hi}CD8^+$ thymocytes (Figures S3 and S4). These results indicate that failure to upregulate surface CD4 expression during differentiation of signaled DP into intermediate thymocytes is sufficient for error-prone MHC II-specific lineage choice.

Because endogenous and transgenic CD4 proteins were transcriptionally regulated by different regulatory elements, the unique upregulation of endogenous CD4 proteins during MHC II-specific positive selection must have been the result of increased *Cd4* gene transcription. To verify that the transcription of endogenous *Cd4* genes in signaled thymocytes increased during MHC II-specific positive selection, we quantified surface

expression of CD4r-encoded hCD2 proteins, which tracks endogenous *Cd4* gene activity. In fact, expression of CD4r-encoded hCD2 proteins was upregulated on signaled TCR^{hi} thymocytes (Figure 2B, lower-left panel), indicating that *Cd4* gene transcription progressively increased in signaled thymocytes throughout MHC II-specific positive selection.

Error-Prone CD4 Lineage Choice and RUNX3 Expression

Because CD8 lineage choice is specifically associated with upregulation of the transcription factor RUNX3 (Egawa et al., 2007; Sato et al., 2005; Setoguchi et al., 2008; Taniuchi et al., 2002), whereas CD4 lineage choice is associated with upregulation of the transcription factors ThPOK (He et al., 2005; Sun et al., 2005), TOX (Aliahmad and Kaye, 2008; Wilkinson et al., 2002), and GATA3 (Hernandez-Hoyos et al., 2003; Wang et al., 2008), we quantified expression of these transcription factors in purified intermediate thymocytes during MHC II-specific positive selection (Figure 3). As expected, expression of the CD4 lineage-associated factors ThPOK, TOX, and GATA3 was upregulated during differentiation of DP into intermediate thymocytes in all mice examined (Figure 3), with the extent of upregulation greater in intermediate thymocytes expressing higher CD4 as a result of quantitatively stronger MHC II-specific positive-selection signaling (Figure 3). In contrast to induction of CD4 lineage-associated factors, MHC II-specific positive selection does not normally induce RUNX3 expression, and we confirmed this point in intermediate thymocytes from *B2m*$^{-/-}$ mice expressing only CD4^{endo} coreceptors (Figure 3). Importantly, however, RUNX3 expression was upregulated in MHC II-selected intermediate thymocytes from mice expressing CD4^{hCD2} or CD4^{endo}+CD4^{T3} coreceptors (Figure 3), i.e., only those mice in which MHC II-specific lineage choice was error prone as revealed by the presence of HSA^{-}TCRβ^{hi}CD8^{+} thymocytes (Figures S3 and S4) and CD8^{+} LN T cells (Figures 2B and 2C; Figure S4). Notably, the relatively low expression of RUNX3 in intermediate thymocytes from mice expressing CD4^{hCD2} or CD4^{endo}+CD4^{T3} coreceptors (Figure 3) is consistent with induction of RUNX3 expression only in those intermediate thymocytes making an erroneous lineage choice. Thus, failure of MHC II-specific positive selection to upregulate surface CD4 expression during differentiation of DP into intermediate thymocytes results in induction of the CD8 lineage-associated factor RUNX3 in intermediate thymocytes and generation of erroneous MHC II-specific CD8^{+} T cells.

CD4 Coreceptor Signal Strength Does Not Affect the Integrity of CD4 Lineage Choice

Next, we examined whether CD4 coreceptor signal strength affected the integrity of CD4 lineage choice, because it has been suggested that CD4 lineage choice is driven by strong coreceptor signals transduced by the CD4 cytosolic tail (Itano et al., 1996). Consequently, we assessed whether modifications to the cytosolic tail of transgenic CD4 coreceptor proteins affected lineage choice. We compared *B2m*$^{-/-}$*Cd4*$^{-/-}$ transgenic mice expressing hCD2-driven CD4 transgenes that encoded re-engineered CD4 proteins containing the cytosolic tail of CD4 (CD4^{hCD2}), CD8α (4aa^{hCD2}), CD8β (4bb^{hCD2}), or no tail at all (44t^{hCD2}) (Figure S5A). Surface CD4 expression was roughly comparable to that of endogenous CD4, with the exception of

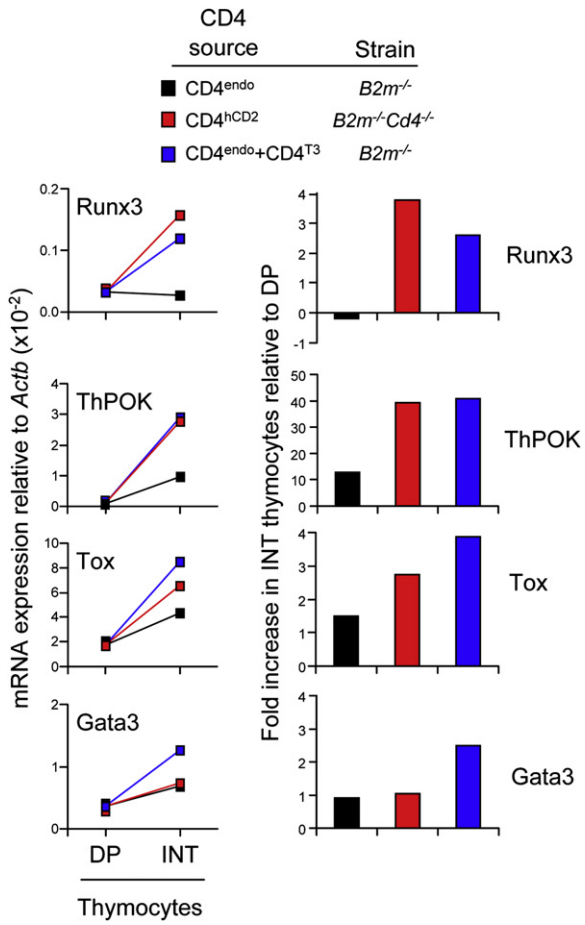

Figure 3. Analysis of Lineage-Specifying Gene Expression during Positive Selection

Expression of mRNAs specific for Runx3, ThPOK, Tox, and Gata3 were assessed in flow-cytometry-sorted preselection DP (CD69^{-}TCRβ^{lo}) thymocytes and intermediate (INT; CD69^{+}TCRβ$^{int/hi}$) thymocytes by quantitative real-time PCR. Gene expression was normalized to *Actb* (β-actin) expression in the same samples. Bar graphs (right) represent the fold increase in mRNA expression between DP and INT thymocytes from the same mice. Data are representative of two independent experiments.

the 44t^{hCD2} transgene, which displayed very high CD4 expression, because tailless CD4 proteins cannot be internalized from the cell surface (Figure S5A). As we previously reported (Van Laethem et al., 2007), the re-engineered CD4 proteins displayed a distinct hierarchy of Lck binding such that coreceptors bearing the CD4 cytosolic tail bound the most Lck, coreceptors bearing the CD8α cytosolic tail bound less Lck, and coreceptors bearing CD8β or no cytosolic tail bound no detectable Lck (Figure S5B). Notably, Lck binding determines CD4 signaling strength and had a substantial quantitative effect on MHC II-specific positive selection, given that the frequency of signaled TCRβ^{hi} thymocytes paralleled the CD4-Lck associations in each strain (CD4^{hCD2} > 4aa^{hCD2} > 4bb^{hCD2} = 44t^{hCD2}) (Figure 4A). However, regardless of CD4 signal strength or overall CD4 expression, MHC II-specific lineage choice was error prone in all of these CD4 transgenic mice, because each contained CD8^{+} LN T cells (Figure 4A, right panels). Contrary to the strength-of-signal

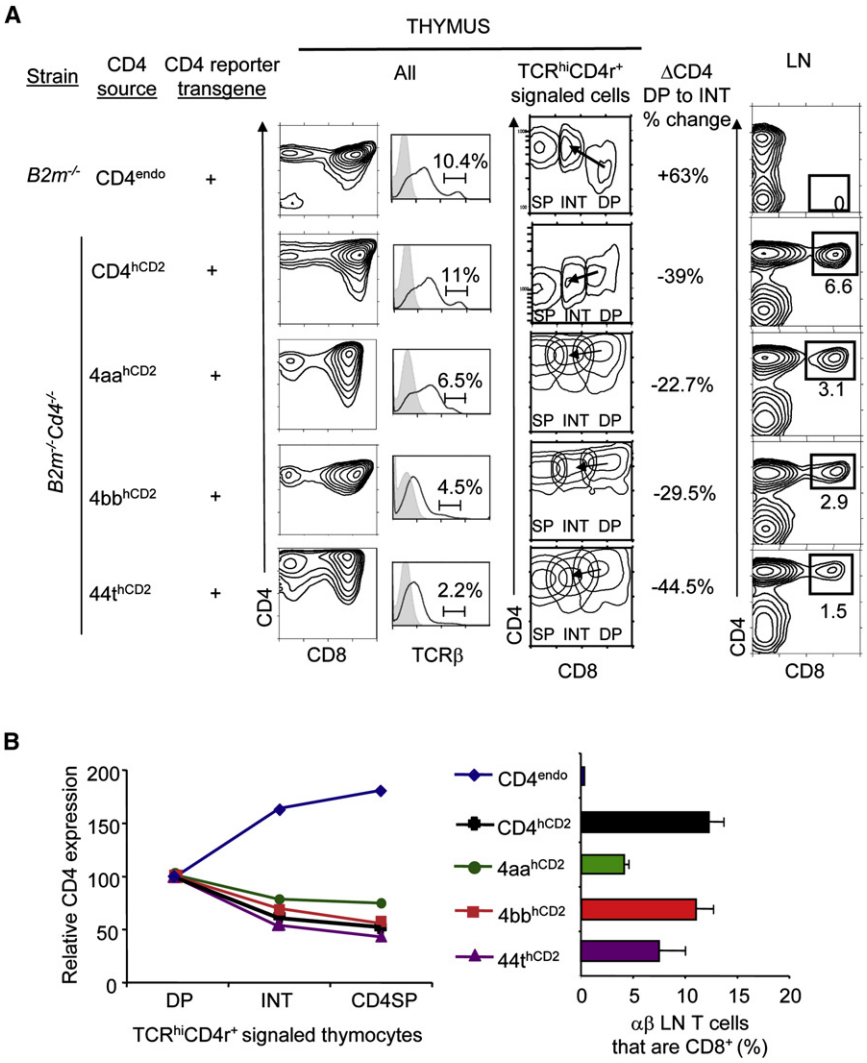

Figure 4. Lineage-Choice Errors Are Unrelated to CD4 Signal Strength

(A) Characterization of thymocytes and T cells in mice expressing wild-type and re-engineered CD4 coreceptor proteins. Re-engineered CD4 transgenes were named according to the origin of their external, transmembrane, and cytosolic domains as 4aa (with transmembrane and cytosolic domains of CD8α), 4bb (with transmembrane and cytosolic domains of CD8β), and 44t (with no cytosolic tail) and were placed under the control of transgenic hCD2 promoter and enhancer elements. Thymocytes from mice expressing the indicated transgenes were stained for CD4, CD8α, and TCRβ, and the frequency of positively selected $TCR\beta^{hi}$ thymocytes is indicated (left panels). CD4 surface expression on signaled DP, INT, and CD4SP thymocyte populations from each mouse strain was analyzed as in Figure 1B, but only the expanded "y" scale view is presented. Relative changes in CD4 surface expression between TCR-signaled DP and intermediate thymocytes were quantitated as % change. Right panels display CD4 versus CD8 staining of LN cells from the same individual mice, with the frequency of $CD8^+$ T cells in the LN of each mouse indicated. Data are representative of three independent experiments.

(B) Lineage choice is affected by CD4 kinetics, not CD4 signal strength. CD4 surface expression on intermediate and CD4SP thymocytes is expressed relative to CD4 expression on signaled ($TCR\beta^{hi}$) DP thymocytes—which was set equal to 100%—from each strain (left panel). Error-prone lineage choice is indicated by the frequency (± SE) of LN T cells that are $CD8^+$ for each strain (right panel). The numbers of mice analyzed per strain were as follows: $CD4^{hCD2}$ = 7, $4aa^{hCD2}$ = 3, $4bb^{hCD2}$ = 4, $44t^{hCD2}$ = 4, and $CD4^{endo}$ n = 10.

perspective (Itano et al., 1996), CD4 lineage choice was not less error prone with stronger-signaling CD4 coreceptors and was not more error prone with weaker-signaling CD4 coreceptors (Figure 4A, right panels as an example of one experiment). The full course of all our experiments revealed that lineage choice was error prone in CD4 transgenic mice regardless of CD4 signal strength (Figure 4B, right panel). We then examined whether CD4 coreceptor expression was up- or downregulated on MHC II-signaled thymocytes in these mice (Figure 4B). In each of these transgenic strains, we found that CD4 expression declined during differentiation of MHC II-signaled DP into intermediate thymocytes (Figure 4B, left) and was associated with CD8 lineage-choice errors (Figure 4B, right). Thus, failure to upregulate CD4 during positive selection caused lineage choice to be error prone, regardless of CD4 signal strength or overall CD4 expression.

Competition for Ligand among TCR Transgenic Thymocytes Exacerbates CD4 Lineage-Choice Errors

Finally, we wished to understand our early observation that lineage choice was especially error prone in $CD4^{hCD2}$ transgenic mice that expressed the AND TCR transgene (AND.$CD4^{hCD2}$ mice) (Figure 1A, bottom panels). Indeed, as compared to $CD4^{hCD2}$ mice with polyclonal TCR, MHC II-specific lineage choice in AND.$CD4^{hCD2}$ mice was excessively error prone, given that nearly 50% of AND T cells in thymus and LN of AND.$CD4^{hCD2}$ mice were mature $CD8^+$ T cells (Figure 5, compare rows 2 and 4). Note that the erroneous differentiation of AND.$CD4^{hCD2}$ thymocytes into $CD8^+$ T cells occurred despite their relatively homogeneous expression of $V\alpha11^{hi}V\beta3^{hi}$ transgenic AND TCR (Figure 5, lower panels). Consequently, we considered that, unlike thymocytes with polyclonal TCR, AND TCR transgenic thymocytes compete with one another for binding to the identical peptide-MHC II-selecting ligand (Huesmann et al., 1991; Wong et al., 2000). However, ligand competition among AND thymocytes, in and of itself, was not an explanation for error-prone lineage choice, because AND thymocytes in mice expressing wild-type $CD4^{endo}$ coreceptors (AND.$CD4^{endo}$) contained no mature $CD8^+$ AND T cells in either thymus or LN (Figure 5, row 2). Nevertheless, we reasoned that AND thymocyte competition for a limiting ligand would exacerbate TCR signaling disruptions caused by downregulation of transgenic CD4 coreceptors, increasing lineage-choice errors. If our reasoning is

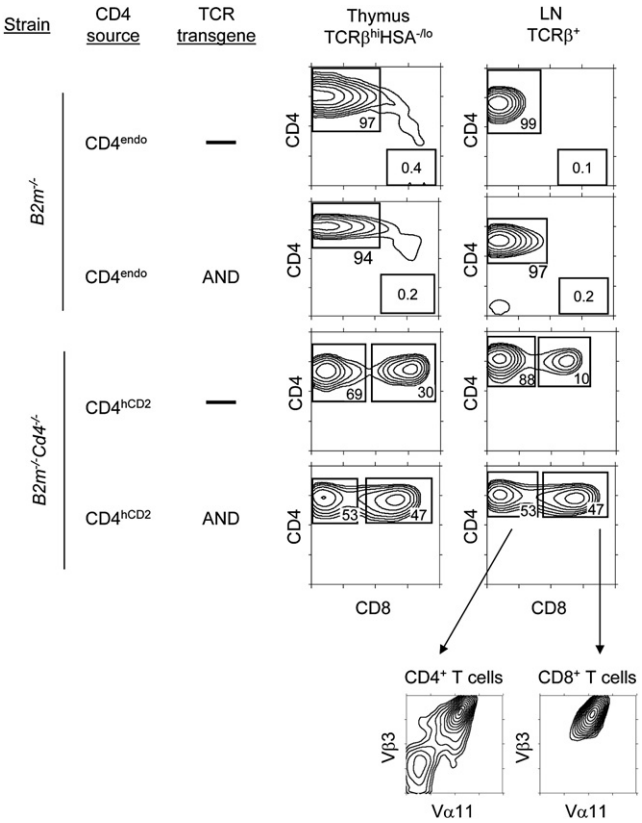

Figure 5. Expression of the AND TCR Exacerbates Lineage-Choice Errors in CD4 Transgenic Mice

CD4 versus CD8 expression of mature, MHC II-selected T cells in the thymus (left panels) and LNs (right panels) of $B2m^{-/-}$ mice expressing endogenous coreceptor proteins or $B2m^{-/-}Cd4^{-/-}$ mice expressing transgenic CD4 coreceptor proteins, and either polyclonal or AND TCR, as indicated. Bottom panels display Vα11 versus Vβ3 expression of gated LN T cells. Boxes identify $CD4^+$ and $CD8^+$ T cells and their relative frequencies. Data are representative of five independent experiments.

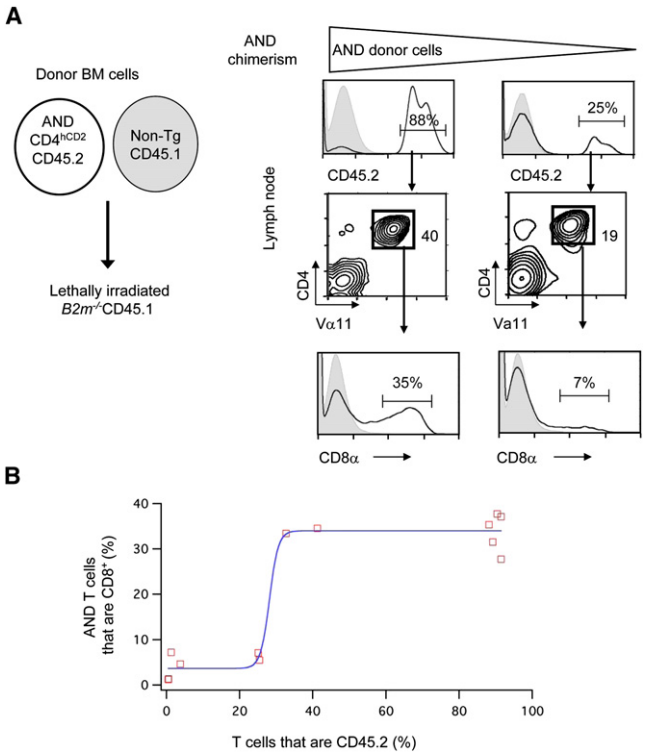

Figure 6. Ligand Competition Increases Lineage-Choice Errors among AND T Cells in $CD4^{hCD2}$ Mice

(A) Mixed-donor radiation bone marrow (bm) chimeras were constructed by reconstituting lethally irradiated (950R) $B2m^{-/-}$ (CD45.1) mice with a total of 10^7 donor bm from $B2m^{-/-}Cd4^{-/-}$AND.$CD4^{hCD2}$ (CD45.2) and $B2m^{-/-}$ (CD45.1) mice, such that AND.$CD4^{hCD2}$ bm constituted 100%, 10%, or 1% of the total bm inoculum. Ten weeks after reconstitution, LN cells were assessed for surface expression of the indicated markers. Data represent five independent experiments.

(B) Effect of ligand competition on error-prone lineage choice. The frequency of donor AND (CD45.2) LN T cells that were $CD8^+$ was plotted against the frequency of AND (CD45.2) T cells in each chimera.

correct, it would then be predicted that decreasing the frequency of cells in an individual thymus that expressed the AND TCR would decrease ligand competition and make AND lineage choice less error prone.

To test this prediction, we constructed radiation bone marrow (bm) chimeras in which lethally irradiated $B2m^{-/-}$ (CD45.1) host mice were reconstituted with donor bm stem cells from $B2m^{-/-}Cd4^{-/-}$AND.$CD4^{hCD2}$ (CD45.2) and nontransgenic $B2m^{-/-}$ (CD45.1) mice in varying ratios so that AND.$CD4^{hCD2}$ (CD45.2) bm cells constituted 100%, 10%, or 1% of the mixed donor bm inoculum (Figure 6A). Ten weeks after bm reconstitution, LN T cells from high frequency AND chimeras were 80%–95% AND (CD45.2), LN T cells from medium-frequency AND chimeras were 20%–45% AND, and LN T cells from low-frequency AND chimeras were 1%–5% AND (Figure 6A and data not shown). The relative frequency of AND T cells that erroneously differentiated into $CD8^+$ T cells was not constant, because the frequency of AND T cells that were $CD8^+$ was relatively high (35%) in chimeras with many AND (CD45.2) T cells but was relatively low (7%) in chimeras with few AND (CD45.2) T cells (Figure 6A). In fact, the lineage error rate among AND.$CD4^{hCD2}$ T cells was relatively low in chimeras in which fewer than 30% of T cells expressed the AND TCR transgene but sharply increased in chimeras in which more than 30% of T cells expressed the AND TCR transgene (Figure 6B). In other words, the frequency of lineage-choice errors among developing AND.$CD4^{hCD2}$ T cells was quantitatively affected by the number of AND.$CD4^{hCD2}$ thymocytes that simultaneously competed for the same intrathymic selecting ligand. Thus, lineage choice is strikingly error prone in AND.$CD4^{hCD2}$ mice because ligand competition exacerbates AND TCR signaling disruptions in thymocytes that downregulate transgenic CD4 coreceptors during MHC II-specific selection (schematized in Figure S6).

DISCUSSION

Kinetic changes in CD8 coreceptor expression during MHC I-specific positive selection are important for CD8 lineage choice, but the importance of CD4 kinetics for CD4 lineage choice has not previously been appreciated. The present study now reveals that transcriptional upregulation of CD4 coreceptor

expression during differentiation of MHC II-signaled DP into $CD4^+CD8^{lo}$ intermediate thymocytes is essential for error-free CD4 lineage choice, especially in TCR transgenic mice whose thymocytes compete for the identical selecting ligand. Indeed, it is the kinetics of CD4 coreceptor expression, not CD4 coreceptor number or signaling strength, that determines the integrity of CD4 lineage choice during MHC II-specific positive selection. Together with current knowledge about the kinetics of CD8 coreceptor expression during positive selection (Kioussis and Ellmeier, 2002; Singer et al., 2008), the present study reveals that TCR-mediated positive-selection signals downregulate CD8 but upregulate CD4 coreceptor expression, disrupting MHC I-specific TCR signaling to promote CD8 lineage choice and prolonging MHC II-specific TCR signaling to promote CD4 lineage choice.

The present study provides a new dimension to the kinetic signaling perspective that CD4 lineage choice requires sustained TCR signaling during differentiation of DP into $CD4^+CD8^{lo}$ intermediate thymocytes. CD4 upregulation during this developmental step stabilizes MHC II-specific TCR-ligand interactions so that TCR signaling persists to drive CD4 lineage choice. In contrast, CD4 downregulation makes it difficult to sustain MHC II-specific TCR-ligand interactions, so that TCR signaling disruptions resulting in lineage-choice errors occur. Our current observation that error-free CD4 lineage choice requires CD4 upregulation regardless of the absolute number or signaling strength of the CD4 coreceptors emphasizes the difference between the TCR signaling requirements for positive selection and the TCR signaling requirements for CD4 lineage choice: the ability of individual DP thymocytes to be signaled by their MHC II-specific TCR to undergo positive selection is affected by the absolute number and signaling strength of the CD4 coreceptors that they express; however, once signaled to undergo positive selection, error-free differentiation into $CD4^+$ T cells requires that CD4 expression further increase to maintain MHC II-specific TCR signaling. In fact, because MHC II-specific TCR-ligand interactions would be especially difficult to sustain in the absence of CD4 coreceptor expression altogether, our present study readily explains why lineage choice among MHC II-signaled thymocytes is so highly error prone in CD4-deficient mice (Matechak et al., 1996; Tyznik et al., 2004).

The present study also provides a new dimension to our understanding of ligand competition during thymic selection by demonstrating that ligand competition can affect the integrity of CD4 lineage choice by MHC II-specific TCR transgenic thymocytes. Indeed, coreceptor kinetics explains why introduction of the AND TCR transgene caused CD4 lineage choice to be strikingly error prone in CD4 transgenic mice, but did not cause CD4 lineage choice to be error prone in CD4 wild-type mice (Wong et al., 2000). In both CD4 transgenic and CD4 wild-type mice, preselection AND thymocytes would compete with one another for the same peptide-MHC II-selecting ligand, and only preselection AND thymocytes that successfully bound its selecting ligand would be signaled to undergo positive selection. Importantly, in CD4 wild-type mice, endogenous CD4 expression is upregulated during positive selection so that signaled AND thymocytes express more endogenous CD4 than unsignaled AND thymocytes, with the result that unsignaled AND thymocytes cannot out-compete signaled AND thymocytes for binding to the selecting ligand. But in CD4 transgenic mice, transgenic CD4 expression is downregulated during positive selection, so signaled AND intermediate thymocytes express less transgenic CD4 than unsignaled preselection AND thymocytes, with the result that unsignaled AND thymocytes out-compete signaled AND thymocytes for binding to the selecting ligand, disrupting AND TCR signaling. Thus, in CD4 wild-type mice, endogenous CD4 upregulation prevents ligand competition among AND thymocytes from introducing lineage-choice errors; however, in CD4 transgenic mice, CD4 downregulation synergizes with ligand competition to make AND lineage choice strikingly error prone.

The $CD4^{T3}$ transgene used in this study was reported in the past to promote generation of MHC II-specific $CD8^+$ T cells and to support the stochastic-selection model of lineage choice (Davis et al., 1993). It was thought that forced transgenic expression of CD4 coreceptors during positive selection rescued from cell death short-lived MHC II-signaled thymocytes that had stochastically made a CD8 lineage choice (Davis et al., 1993). However, this explanation and the presumptions underlying the stochastic-selection model have since been experimentally disproved (Adoro et al., 2008; Dave et al., 1998; Itano and Robey, 2000; Sarafova et al., 2005; Singer et al., 2008), leaving the generation of MHC II-specific $CD8^+$ T cells in $CD4^{T3}$ transgenic mice unexplained. The present study now indicates that MHC II-specific lineage choice is error prone in $CD4^{T3}$ and other CD4 transgenic mice because expression of transgenic CD4 coreceptors fails to be upregulated during MHC II-specific positive selection, which leads to TCR signaling disruptions and lineage-choice errors. Similarly, the appearance of MHC II-selected $CD8^+$ T cells in CD4-silencer-deficient mice was also originally explained as revealing a stochastic mechanism underlying lineage choice (Leung et al., 2001). However, deletion of the CD4 silencer element additionally deleted cryptic CD4 enhancer elements transcriptionally active in signaled thymocytes, because CD4 coreceptor expression on MHC II-signaled thymocytes in CD4 silencer-deficient mice was reduced relative to wild-type mice (Leung et al., 2001). In fact, careful examination of published thymocyte profiles from these CD4 silencer-deficient mice reveal that CD4 coreceptor expression dramatically declined on signaled DP thymocytes during their differentiation into $CD4^+8^{lo}$ intermediate thymocytes, explaining why MHC II-specific lineage choice was error prone in these animals.

Expression of endogenously encoded CD4 coreceptors is transcriptionally upregulated during MHC II-specific selection, but the molecular mechanism by which this occurs is not yet clear. Transcriptional regulation of the endogenouos *Cd4* gene locus still remains incompletely understood (Ellmeier et al., 1999; Leung et al., 2001), but we suspect that *Cd4* enhancer elements exist that increase *Cd4* gene transcription in response to TCR-mediated positive-selection signals. Even though the existence of TCR-responsive enhancer elements in the *Cd4* gene remain a matter of speculation, the transcription factor Tox appears to be necessary for increased *Cd4* gene expression during positive selection, given that CD4 expression on signaled thymocytes from Tox-deficient mice is downregulated, rather than upregulated, during MHC II-specific selection (Aliahmad and Kaye, 2008).

Finally, our conclusion that error-free CD4 lineage choice requires CD4 upregulation to sustain MHC II-specific TCR

signaling conflicts directly with the concept that, during positive selection, TCR-signaled $CD4^{hi}CD8^{hi}$ (DP^{hi}) thymocytes downregulate expression of both CD4 and CD8 coreceptors to become $CD4^{lo}CD8^{lo}$ (DP^{lo}) thymocytes, which then differentiate into $CD4^{+}CD8^{lo}$ intermediate cells that ultimately differentiate into either $CD4^{+}$ or $CD8^{+}$ T cells (Aliahmad and Kaye, 2008; He and Kappes, 2006; Lucas and Germain, 1996; Sant'Angelo et al., 1998). However, precursor-progeny assessments have never actually documented that $CD4^{+}$ T cells arise from DP^{lo} thymocytes that had previously been DP^{hi}, and we do not think that this is the phenotypic pathway by which mature $CD4^{+}$ T cells arise. Rather, we think that CD4 coreceptor expression is constantly increasing on MHC II-signaled thymocytes, progressively increasing the stability of MHC II-specific TCR-ligand interactions and promoting error-free CD4 lineage choice. That is, we think that MHC II-specific TCR signals induce newly arising DP^{lo} thymocytes to upregulate CD4 (and terminate CD8) expression to become $CD4^{+}8^{lo}$ intermediate thymocytes, which then differentiate into mature $CD4^{+}$ T cells. Consequently, we consider DP^{hi} thymocytes to be cells that have failed MHC II-specific positive selection but might still be signaled by MHC I ligands to differentiate into $CD8^{+}$ T cells.

In conclusion, the kinetics of CD4 coreceptor expression during MHC II-specific positive selection importantly influence the integrity of CD4 lineage choice, especially in TCR transgenic mice whose thymocytes compete for limiting ligand. Building on current knowledge about the kinetics of CD8 coreceptor expression, this study reveals that coreceptor kinetics during positive selection promote error-free CD8 lineage choice by disrupting MHC I-specific TCR signaling and promote error-free CD4 lineage choice by prolonging MHC II-specific TCR signaling.

EXPERIMENTAL PROCEDURES

Animals and Transgenic Constructs

C57BL/6 (B6), *B2m*$^{-/-}$, and *Cd4*$^{-/-}$ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). *B2m*$^{-/-}$*Cd4*$^{-/-}$ double-deficient mice were generated and bred to mice expressing the line 30 CD4 reporter transgene (CD4r) from D. Littman (NYU) (Sawada et al., 1994). All CD4 transgenes in the present study were expressed in *B2m*$^{-/-}$*Cd4*$^{-/-}$$CD4r^{+}$ unless otherwise indicated. The 8DP4 transgene has previously been characterized (Sarafova et al., 2005) and expresses CD4 under the control of $E8_{III}CD8\alpha$ enhancer and $CD8\alpha$ promoter elements (Ellmeier et al., 1998). $CD4^{T3}$ transgenic mice express CD4 under the control of hCD3δ promoter-enhancer elements and were constructed in our laboratory from a transgenic vector kindly provided by D. Littman (Davis et al., 1993). The $CD4^{hCD2}$ transgene expresses CD4 under the control of hCD2 promoter and enhancer elements, as previously described (Van Laethem et al., 2007). A brief description of the CD4 transgenic mice used in this study is provided (Table S1). Where indicated, mice also expressed the AND TCR transgene (Kaye et al., 1989). Mixed radiation bm chimeras were constructed by reconstituting 950R irradiated host mice with a total of 10^{7} T-depleted bm cells from CD45.1 and CD45.2 donor mice. All mice were cared for in accordance with National Institutes of Health (NIH) guidelines and with the approval of the National Cancer Institute animal care and use committee.

CD4 Transgenic Mice Expressing Re-engineered CD4 Proteins with Different Cytosolic Tails

Re-engineered CD4 coreceptor proteins expressing different cytosolic tails were generated from cDNAs encoding CD4, $CD8\alpha$, and $CD8\beta$ by conventional cloning procedures. Amino acid sequences at the modified junctions were as follows: 4aa ext/tm junction, VNQT/DIYIWAPLAGIC; 4bb ext/tm junction, VNQT/DITTLSLL; and 44t tm/cyto junction, GLCILCCV/RCRHQQRQ. The resulting chimeric cDNAs were inserted into the hCD2-based cassette and were used to generate transgenic mice, as previously described (Van Laethem et al., 2007). Transgenic offspring were mated to *B2m*$^{-/-}$*Cd4*$^{-/-}$ mice or to AND TCR transgenic *B2m*$^{-/-}$*Cd4*$^{-/-}$ mice. Transgenic offspring were identified by the presence of $CD4^{+}CD8^{+}$ cells in the blood. The absence of endogenous CD4 and β2m were confirmed by PCR. All mice in this study were heterozygous for the transgene(s) they expressed.

Antibodies

Monoclonal antibodies with the following specificities were used in this study: CD3 (145-2C11 PharMingen), CD4 (GK1.5, PharMingen), $CD8\alpha$ (CT-CD8 α, Caltag), CD24 (M1-69, PharMingen), CD69 (H1.2F3, PharMingen), hCD2 (CD0215-4, Caltag), $H\text{-}2K^{b}$ (AF6-88.5, PharMingen), TCRβ (H57-597, PharMingen), CD45.1 (A20, PharMingen), CD45.2 (104, PharMingen), TCR-Vβ3 (KJ25, PharMingen), and TCR-Vα11 (RR8-1, PharMingen).

Flow Cytometry

CD4 fluorescence on TCR-signaled DP thymocytes (identified as $TCR\beta^{hi}CD4r^{+}CD8^{hi}$ cells), intermediate thymocytes (identified as $TCR\beta^{hi}CD4r^{+}CD8^{int}$ cells), and CD4SP thymocytes (identified as $TCR\beta^{hi}CD4r^{+}CD8^{-}$ cells) was either expressed as mean fluorescence intensity (MFI) or quantitated into linear total fluorescence units (TFUs) with an empirically derived calibration curve constructed for each logarithmic amplifier. Relative CD4 expression on each cell population was calculated relative to signaled DP thymocytes. To avoid fluorescence compensation errors that might have affected our flow-cytometric data, we verified that CD4 versus CD8 profiles from samples stained with only two colors were always identical to CD4 versus CD8 profiles from multicolor stained samples. In addition, three different fluorochrome combinations were used for TCR versus CD4 versus CD8 staining so that results were reproducible on different machines with different lasers and different fluorochromes.

Immunoprecipitation and Immunoblotting

Thymocytes (2×10^{7} cells/group) were solubilized in 1% Brij96, and CD4 molecules were immunoprecipitated with purified anti-CD4 mAb (GK1.5, PharMingen) and protein G-sepharose beads. Whole-cell lysates and immunoprecipitates were resolved by SDS-PAGE on 10% acrylamide (Invitrogen) under reducing conditions and transferred to nitrocellulose membranes (Amersham). Blots were incubated with anti-Lck (3A5, PharMingen) and anti-actin antibodies followed by horseradish peroxidase-conjugated protein A. CD4 protein was detected with RM4.5 rat mAb (PharMingen) followed by horseradish peroxidase-conjugated goat anti-rat antibodies. Reactivity was detected by enhanced chemiluminescence.

Quantitative Real-Time PCR

Preselection DP ($CD69^{-}TCR\beta^{lo}CD4^{+}CD8^{+}$) and intermediate ($CD69^{+}TCR\beta^{int/hi}CD4^{+}CD8^{lo}$) thymocytes were obtained by electronic sorting of thymocyte suspensions, and total RNA was immediately isolated with the RNEasy kit (QIAGEN). RNA was reverse transcribed into cDNA by oligo(dT) priming with the SuperScript III First Strand Synthesis System (Invitrogen). Quantitative RT-PCR (qRT-PCR) was performed with an ABI PRISM 7900HT Sequence Detection System and the SYBR Green detection system (QIAGEN) with the following primers: *Gata3* (F: 5′-GTCCTCATCTCTTCACCTTCC-3′; R: 5′-GAGTCCGCAGGCATTGCAAAG-3′), *Tox* (F: 5′-CAGGACCCCTACTATTGCAAC-3′; R: 5′-GCAGGCCATTGTGATTCATGG-3′), *ThPOK* (F: 5′- ACATGAGGACCCACACTGGTG-3′; 5′-CTTCCTCTTCCTCCTCCTCAG-3′), *Runx3* (F: 5′-GCGACATGGCTTCCAACAGC-3′; R: 5′-CTTAGCGCGCCGCTGTTCTCGC-3′), and *Actb* (F: 5′-GAGAGGGAAATCGTGCGTGA-3′; R: 5′-ACATCTGCTGGAAGGTGGAC-3′). Gene expression values were normalized to those of *Actb* (β-actin gene) in the same sample.

SUPPLEMENTAL DATA

Supplemental Data include seven figures and one table and can be found with this article online at http://www.cell.com/immunity/supplemental/S1074-7613(09)00375-6.

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